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(54) Title: DIAGNOSIS AND TREATMENT OF PATHOLOGICAL PREGNANCIES

(57) Abstract

Disclosed is a method for treating a hypertensive disorder in a pregnant woman, the method comprising administering to the pregnant woman an amount of a therapeutic substance which regulates the amount, and/or activity, of VEGF in the woman effective to ameliorate the hypertensive disorder, together with diagnostic methods and kits.

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Title: Diagnosis and Treatment of Pathological Pregnancies

Related Applications

This Application claims the benefit of US Provisional Application No. 60/057,055, filed 27th August 1997.

Field of the Invention

This invention relates, *inter alia*, to a method of diagnosing hypertensive disorders in pregnant women, and methods of treating such conditions.

Background of the Invention

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a disulfide-linked homodimeric protein of between 24-46 kD with five differentially spliced forms (Houck *et al.*, 1991 Mol. Endocrinol. **5**, 1806-1814; Charnock-Jones, *et al.*, 1993 Biology of Reproduction **48**, 1120-1128). It is a potent stimulator of angiogenesis (i.e. growth of blood vessels) in both the chick chorioallantoic membrane and rabbit cornea assays (Connolly *et al.*, 1989 J. Clin. Invest. **84**, 1470-1478) and promotes endothelial cell proliferation and migration. VEGF is widely expressed in human adult tissues and may play a role in the maintenance of the endothelium (Alon *et al.*, 1995 Nature Medicine **1**, 1024-1028). It is over-expressed in a variety of pathological conditions, particularly solid tumors, whose growth can be prevented by the inhibition of VEGF action (Kim *et al.*, 1993 Nature **362**, 841-844; Millauer *et al.*, 1994 Nature **367**, 576-579). Transgenic mice bearing heterozygous and homozygous deletions of the VEGF gene fail to survive beyond embryonic day 12, showing that VEGF is essential for embryonic development (Ferrara *et al.*, 1996 Nature **380**, 439-442; Carmeliet *et al.*, 1996 Nature **380**, 435-439) and that other closely related growth factors (Joukov *et al.*, 1996 EMBO J. **15**, 290-298; Olofsson *et al.*, 1996 Proc. Natl. Acad. Sci. USA **93**, 2576-2581;

Conn *et al.*, 1990 Proc. Natl. Acad. Sci. USA **87**, 2628-2632) cannot compensate for even its partial loss.

The known receptors for VEGF, the fms-like tyrosine kinase, flt-1 (Shibuya *et al.*, 1990 Oncogene **5**, 519-524); and the kinase domain receptor, KDR (Terman *et al.*, 1992 Biochem. & Biophys. Res. Comm. **187**, 1579-1586), are restricted in their expression patterns. While both receptors are present on endothelial cells obtained from many different tissues (Peters 1993 Dev. Biol. **90**, 8915-8919; Millauer *et al.*, 1991 Biochem. Biophys. Res. Comm. **175**, 68-76; Olander *et al.*, 1991 Biochem. Biophys. Res. Comm. **175**, 68-76), only flt-1 (not KDR) is highly expressed by trophoblast cells (Charnock-Jones *et al.*, 1994 Biol. Reprod. **51**, 524-530; Clark *et al.*, 1996 Hum. Reprod. **11**, 1090-1098). Monocytes and peritoneal fluid macrophages respond to VEGF (Clauss *et al.*, 1990 J. Exp. Med. **172**, 1535-1545; McLaren *et al.*, 1996 J. Clin. Invest. **98**, 482-489) and melanoma and some ovarian tumours have also been shown to express VEGF receptors (Potgens, *et al.*, 1995 Am. J. Pathol. **146**, 197-209; Boocock, *et al.*, 1995 J. Natl. Can. Inst. **78**, 506-516).

Angiogenesis is a complex process necessitating the interaction of numerous cell types which leads to the co-ordinated development of a complex vascular 3-D structure. there are many factors involved in angiogenesis and it is the balance of stimulators such as VEGF and inhibitors like angiostatin that determines the end result (O'Reilly, *et al.*, 1994 Cell **79**, 315-328; Hanahan & Folkman 1996 Cell **86**, 353-364). In the placenta there is profound angiogenesis as high capacity transport between the maternal and the growing fetal circulation develops. However, in the human, it is notable that little angiogenesis occurs in the maternal tissue while there is obvious capillary growth within the placental villi. This suggests that there are locally acting factors which regulate vascular growth.

It has been demonstrated that VEGF is highly expressed in the placenta and that early in gestation maternal macrophages within the decidua contain large amounts of VEGF mRNA (Sharkey *et al.*, 1995 Int. J. Biochem. Cell Biol. **28**, 81-89; Charnock-Jones, *et al.*, 1994 Biol. Reprod. **51**, 524-530). It has also been shown that villous and extravillous trophoblast contain very high levels of mRNA encoding flt-1 (Charnock-Jones, *et al.*, 1994

Biol. Reprod. 51, 524-530; Clark, *et al.*, 1996 Hum. Reprod. 11, 1090-1098).

As well as full-length membrane-bound cell surface receptors for VEGF, there have also been shown to exist *in vitro* truncated forms of flt. These truncated forms of flt arise by variant splicing of the flt mRNA and lack the transmembrane domain, and so are secreted from cells in soluble form (called "sflt", or soluble flt). Such soluble receptors which retain binding affinity for VEGF may act as VEGF-antagonists, as they can compete with the cell-surface receptors for binding to VEGF.

For example, Kendall & Thomas (1993, cited above) showed that a very low level of mRNA (2 clones detected out of $\approx 10^6$) encoding soluble flt could be detected in human endothelial cells, implying that, if the corresponding translation product was even synthesised *in vivo* (which has not hitherto been reported), the level of expression must be very low.

It has also been demonstrated that a VEGF-binding factor is present in the serum of pregnant women (Charnock-Jones *et al.*, Trophoblast Conference, Banff, Canada 1996). There has also been one report that levels of circulatory VEGF in patients complicated by pre-eclampsia are significantly elevated compared to those in control patients (Charnock-Jones *et al.*, Abstract 211, Abstracts of the 10th International Conference on Prostaglandins and Related Compounds, Vienna, September 1996). However there has never been any suggestion that this reflects a causal relationship. Indeed, there is a conflicting report (Lyall *et al.*, International Society for the Study of Hypertension in Pregnancy, 10th World Congress, Seattle USA, August 1996) indicating that VEGF levels are lower than normal in patients with pre-eclampsia. Thus the person skilled in the art is presented with confusing and incomplete knowledge.

The maternal syndrome of pre-eclampsia is characterised by hypertension and proteinuria. There are also a variety of histological changes detectable in the patients. For example, Shankling & Sibai (Ultrastructural Aspects of Pre-eclampsia 1. Placental bed and uterine boundary vessels; American Journal of Obstetrics and Gynaecology 1989 **161**, 735-741) carried out a detailed electron microscopic analysis of maternal vessels in patients with and

without pre-eclampsia. Their major finding was that there was extensive endothelial cell injury, particularly in placental venules where there was some fibrin deposition. In normal pregnancies the invasion of the decidual arterioles by extravillous trophoblast transforms them into high capacity, low resistance vessels. This does not occur in pre-eclampsia, since the trophoblast does not invade sufficiently far into the decidua. Another clinical feature of pre-eclampsia is increased incidence of disseminated intravascular coagulation (DIC). It is thought this may arise from increased fibrin deposition, mainly in the placenta (Dewhurst Textbook of Obstetrics and Gynaecology for Postgraduates, p209 & 221, publ. Blackwell Scientific Publications). Pre-eclampsia is also characterised by a reduction in birth-weight of the infant.

Summary of the Invention

In a first aspect the invention provides a method for treating a hypertensive disorder in a pregnant woman, the method comprising administering to the pregnant woman an amount of a therapeutic substance which regulates the amount, and/or activity, of VEGF in the woman effective to ameliorate the hypertensive disorder.

Typically the hypertensive disorder to be treated by the method of the invention is pre-eclampsia, a very serious pathology associated with foetal and/or maternal morbidity or mortality. There has been a long-felt need for an effective treatment of the condition, but no such treatment has yet been found, despite strenous efforts by many researchers working in this field.

The "activity" of VEGF is herein defined as that set of characteristics which enable abnormalities in VEGF levels to cause pathological conditions (e.g. the ability to bind in a specific manner to cell-membrane VEGF receptors, so as to cause angiogenesis in susceptible tissues). VEGF activity therefore encompasses such characteristics as stimulation of angiogenesis (growth and formation of blood vessels), promotion of endothelial cell proliferation and migration, increased vascular permeability, increased protease and von Willebrand factor production, and tissue factor induction.

The present inventors have found that abnormalities in the level of VEGF activity are of critical importance in the development of the symptoms of pre-eclampsia such that the method of the invention can have a substantial therapeutic effect, either by adjusting the amount of active VEGF in the patient, and/or adjusting the activity thereof, to normal levels. In general, the inventors consider that a therapeutic effect will best be obtained by reducing VEGF amounts and/or activity in the patient, rather than increasing the same.

Those skilled in the art will appreciate that a number of mechanisms are available by which the amount and/or activity of VEGF can be regulated in a patient. Two general strategies for down-regulation include:-

A) Preventing binding of VEGF to cell-membrane receptors on target cells; and
B) Inhibiting one or more of the chain of events which follow binding of VEGF to its receptor.

Within the two categories, numerous possibilities exist. Under heading "A", one could envisage methods which involve use of substances which bind to the VEGF receptor (but do not cause activation thereof) and so prevent VEGF binding, or use of substances which bind to VEGF and prevent it binding to its receptor. Under heading "B", one could envisage use of molecules which inhibit the tyrosine kinase activity of the VEGF receptor.

Such substances might comprise nucleic acids (especially short RNA molecules), polypeptides and peptides (naturally-occurring or synthetic) or synthetic compounds.

Those skilled in the art will appreciate that, for methods of down-regulating VEGF activity in the patient which comprise the use of a competitive inhibitor to prevent VEGF binding to its receptor, it will be preferred that the affinity of the inhibitor for VEGF, or for VEGF receptor (as appropriate), will be comparable to the binding affinity between VEGF and its FLT-1 receptor, which affinity is approximately 10-20 picomolar. Those skilled in the art will however also appreciate that lower binding affinities of inhibitors can be compensated by the administration of large amounts of inhibitor, so as to create a comparatively high concentration of inhibitor in the patient relative to the concentration

of VEGF, or VEGF receptor, as appropriate, such that the inhibitor competes effectively with VEGF.

In one embodiment the method of treatment in accordance with the invention comprises administering a therapeutic substance which down-regulates (i.e. reduces) the amount and/or activity of free VEGF in the patient, preferably to, or slightly below, the level of free VEGF associated with a normal pregnancy. Those skilled in the art will appreciate that it may not be necessary to reduce that total amount of VEGF in the patient, but that a therapeutic effect may be obtained by reducing the amount of free VEGF in the circulation of the patient.

By way of explanation "free VEGF" is the amount of uncomplexed VEGF (not bound to receptor) in the patient. It is possible that VEGF binds weakly in the circulation to albumen. Such interaction, if it occurs, is thoght to be quite weak and does not appear likely to affect the activity of the VEGF. Accordingly VEGF bound weakly to albumen or similar plasma protein is considered for present purposes as uncomplexed, and thus "free VEGF". Conveniently, a measure of the amount of free VEGF can be made by assaying VEGF levels in the serum of the patient.

The invention encompasses within its scope the administration of a substance which antagonizes VEGF, either by reducing the amount of VEGF available to its receptor, competitively inhibiting VEGF from binding to its receptor, or inhibiting the tyrosine kinase activity of VEGF.

Substances which are useful according to the invention include soluble VEGF receptors (such as sflt-K, or the soluble flt variants disclosed by Charnock-Jones *et al*, in PCT/GB95/01213). Charnock-Jones *et al* disclose soluble forms of the flt polypeptide that are capable of binding to VEGF and exerting an inhibitor effect thereon, the polypeptide including five or fewer complete immunoglobulin-like domains. The soluble forms do not contain a transmembrane domain. Two such soluble forms are FLT4 and FLT15. Both include at their N-terminus the amino acid sequence corresponding to the equivalent portion of the unaltered wild-type flt polypeptide (i.e. 440-480 amino acids of N-terminal

flt sequence) followed by a 65 amino acid polypeptide (FLT4) or a 39 amino acid polypeptide (FLT15). The sequences of FLT4 and FLT15 are provided in Charnock-Jones *et al*. Aiello *et al* (1995 Proc. Natl. Acad. Sci. USA 92, 10457-10461) demonstrated the efficacy of a soluble receptor as a VEGF antagonist in mice in inhibiting angiogenesis.

Another soluble antagonist of VEGF is Placental Growth Factor (PlGF), which binds to the FLT-1 receptor molecule. Accordingly a therapeutic substance in accordance with the invention may comprise PlGF, or a portion thereof which retains specific binding activity for FLT-1.

As VEGF and PlGF both bind to FLT and to soluble forms of FLT, the inventors suggest that administration of a substance which regulates the balance between VEGF, PlGF and FLT may have a therapeutic effect. Thus, for example, administration of PlGF or SFLT may have a beneficial effect, as described above, and similarly administration of other substances which affect the level and/or activity of VEGF, PlGF or FLT may also be effective.

Other soluble substances with suitable VEGF-antagonistic activity include anti-VEGF immunoglobulin molecules (or effective portions thereof), that is, having specific binding affinity for VEGF. Examples of effective portions of immunoglobulin molecules include scFv, Fv, Fab and Fab_2 fragments, all of which are known to those skilled in the art. The immunoglobulin molecule or portion thereof may be a naturally-occurring or engineered antibody (e.g. chimeric antibody, "humanised" antibody and the like) such as those taught by PCT/US92/09218.

Alternatively, the soluble VEGF antagonist may be a chimeric polypeptide (e.g. comprising a VEGF-binding portion of a VEGF receptor, fused to another moiety, such as an immunoglobulin). Aiello *et al* describe one molecule containing the entire extracellular domain (758 residues) of the human high affinity VEGF receptor flk-1 fused to the sequence coding for amino acids 216-443 of the human IgGγ1 heavy chain. Equally, the VEGF antagonist may be a chimeric polypeptide comprising an immunoglobulin molecule, or an effective portion thereof, having specific binding activity

for VEGF or for the VEGF receptor FLT-1 or KDR.

Other representative examples of substances according to the invention include truncated forms of a receptor in which the transmembrane and cytoplasmic domains are deleted from the receptor, and fusion proteins in which non-human VEGF receptor polymers or polypeptides are conjugated to the human VEGF receptor (hVEGFr) or truncated forms thereof. An example of such a non-hVEGF polypeptide is an immunoglobulin. The extracellular domain of the hVEGFr is substituted for the Fv domain of an immunoglobulin light or heavy chain, with the C-terminus of the receptor extracellular domain covalently joined to the amino terminus of the Ch1, hinge, CH2 or other fragment of the heavy chain. Such variants are made in the same fashion as known immunoadhesons. See, e.g. Gascoigne *et al*, PNAS 84:2936, 1987; Capon *et al*, Nature 337:525, 1989; Aruffo *et al*, Cell 61:1303, 1990; Ashkenazi *et al*, PNAS 88:10535, 1991; and Bennett *et al*, Jour. Biol. Chem., 266:23060, 1991. In other substances according to the invention, the hVEGFr is conjugated to a nonproteinaceous polymer such as PEG (Davis *et al*, USP 4,179,337; Goodson *et al*, Biotech. 8:343, 1990). This serves to extend the biological half-life of the hVEGFr and reduces the possibility that the receptor will be immunogenic. HVEGFr is used substantially in the same fashion as antibodies to hVEGF, taking into account the affinity of the antagonist and its valency for hVEGF.

The optimum dose administered to a patient in need of treatment according to the invention will depend on the efficacy of the substance in question.

The amount to be administered may be determined by measuring the resultant increase or decrease in VEGF relative to a normal level of VEGF (i.e. found in a healthy pregnant woman) or it may be determined by monitoring the hypertensive condition, i.e., looking for a reduction in symptoms associated with hypertension and pre-eclampsia. An "effective amount" of a substance which reduces or increases the VEGF level or activity in a patient is that amount which is effective to bring VEGF to a normal level or to reduce symptoms of hypertension in a pregnant woman or to reduce the symptoms of pre-eclampsia.

Generally, the dosage of peptide (naturally occurring or synthetic) or polypeptide will be in the range of 100ng - 10μg/kg body weight. Where the substance administered is a nucleic acid, such as an RNA molecule, the dosage is in the range of 10ng-1μg/kg body weight.

As described above, measurement of VEGF levels may be useful in monitoring the efficacy of treatment. Reduction in the amount of VEGF available to its receptor may involve measuring the physiological level of VEGF and administering the substance in an amount sufficient to reduce the VEGF level to a level which is normal in a pregnant woman, i.e. a level which does not result in hypertension. As used herein, a "normal" level (or amount) of VEGF is dependent upon the assay method employed to measure VEGF. Baker *et al*, (1995, Obstetrics & Gynaecology 86:815) measure VEGF in a healthy individual at 5.5pmol/L; Sharkey *et al* (1996, Eur. Jour. Clin. Invest. 26:1182) measure VEGF in the range of 6.3-24.3 ng/ml; and Lyell *et al*, (1997, Obstetrics & Gynaecology 104) measure VEGF in the range of 1.8-450 pg/ml. The Baker *et al* method, 1995, supra, is as follows.

Serum samples are collected from a pregnant woman and maintained at 26°C before centrifugation at 2000 x g for 20 min, then aliquoted under sterile conditions and stored at -80°C. Levels of VEGF in the sample were measured using a modification of the human VEGF assay described by Yeo *et al*, (1992, Clin. Chem. 98:71), hereby incorporated by reference. In brief, rabbit polyclonal antibodies developed against recombinant human VEGF are used as both the capture and detection antibodies. Affinity-purified anti-VEGF IgG is labelled with Eu+3-chelate and used as second antibodies. In the presence of VEGF, a sandwich is formed, and after a final wash, Eu+3 is dissociated from second antibodies with an enhancement buffer containing β-diketone (Pharmacia, Gaithersburg, MD) to produce a highly fluorescent chelate that was measured in a time-resolved fluorimeter. Human recombinant VEGF purchased from R&D systems (Mpls, MN) was used to calibrate the assay. The lower limit of detection of VEGF (+3 SDs of the zero standard) was 5.5 pmol/L.

Those skilled in the art, e.g., a medical doctor, will be able to determine an appropriate

dosage based on the degree of hypertension exhibited by the patient. These symptoms include but are not limited to proteinuria and hyperuricemia.

"Hypertension" is herein defined as an increase of 30 mmHg systolic or 15 mmHg diastolic blood pressure, compared with values obtained before 20 week's gestation, or an absolute blood pressure of at least 140/90 mmHg after 20 weeks if blood pressure recordings in the first half of pregnancy are unknown. "Proteinuria" is herein defined as more than 500 mg per 24 hour collection, or at least 2+ on a voided or at least 1+ on a catheterized random urine specimen. "Hyperuricemia" is herein defined as at least one standard deviation above the normal mean concentration (5.5 mg/dl at term).

The therapeutic substance will generally be delivered by injection, mixed in a composition comprising a physiologically acceptable carrier, such as sterile saline solution and the like. In other embodiments the therapeutic substance may be administered by intra-venous drip, as most patients with pre-eclampsia are hospitalized.

Those skilled in the art will appreciate that, as an alternative to direct introduction into the patient of a substance which affects the level of free VEGF, it may be possible to introduce into the patient a nucleic acid directing the expression of a polypeptide product which has the desired therapeutic effect (a "gene therapy" technique), although the former method will generally be preferred as being simpler and more convenient.

In a second aspect the invention provides for use of a therapeutic substance, which regulates the amount and/or activity of VEGF in a human subject, in the manufacture of a medicament to treat a hypertensive disorder in a pregnant woman. As explained above, the therapeutic substance conveniently causes a down-regulation in VEGF levels/activity (e.g. may be a VEGF antagonist), and the medicament is typically used to treat pre-eclampsia. The medicament will comprise one or more of the therapeutic substances defined above in connection with the first aspect of the invention.

In a third aspect the invention provides a method of diagnosing a hypertensive disorder (particularly, pre-eclampsia) in a pregnant woman, the method comprising obtaining a

sample of body fluid from the patient and determining, either qualitatively or quantitatively, the amount of VEGF in the sample. Typically the sample of body fluid will comprise a blood or urine sample. Conveniently the method will comprise a binding assay to measure the amount of VEGF in the sample, and will comprise the use of a binding partner having specific binding activity for VEGF (such as a VEGF receptor, or an anti-VEGF immunoglobulin molecule or portion thereof). Suitable assays are known to those skilled in the art and include those working on the principles of ELISA, RIA, Western blotting etc. In particular, suitable kits are commercially available and obtainable, for example, from Peninsular Laboratories, USA (a competitive enzyme inhibition assay) or R & D Laboratories, Abingdon, Oxford (an ELISA test).

In a fourth aspect, the invention provides a method of diagnosing a hypertensive disorder (particularly pre-eclampsia) in a pregnant woman, the method comprising obtaining a sample of body fluid from the woman and determining, either qualitatively or quantitatively, the amount of soluble VEGF receptor (e.g. sflt) in the sample. As in the third aspect of the invention, the sample of body fluid conveniently comprises a blood or urine sample, and the amount of soluble receptor will conveniently be determined by a specific binding assay. Again, suitable binding assays will be apparent to those skilled in the art.

Preferably, the same body fluid sample taken from the patient may be divided into portions and respective portions used to determine the amount of VEGF and the amount of soluble receptor.

Further by way of explanation, the present inventors have surprisingly found that soluble flt (sflt) is produced in large amounts by the placenta in women with normal pregnancies, and that sflt can be detected in the serum of such women. Conversely, it is predicted that women with pre-eclampsia produce insufficient amounts of sflt, leading to the presence of excessive amounts of free, active VEGF.

The diagnostic methods of the third and fourth aspects of the invention will conveniently be performed in conjunction with other tests for diagnostic indicators (e.g. measuring

blood pressure).

In a fifth aspect, the invention provides a method of diagnosing a hypertensive disorder (particularly pre-eclampsia) in a pregnant woman, the method comprising obtaining a sample of body fluid (e.g. blood or urine) from the woman and determining, either qualitatively or quantitatively, the amount of Placental Growth Factor (PlGF) in the sample.

By way of explanation, the present inventors have found a statistically extremely significant correlation between the amount of PlGF in the serum and development of hypertension during pregnancy: in normotensive pregnant women, the average amount of PlGF in the serum is about 500pg/ml. In contrast, in pre-eclamptic pregnant women the average concentration of PlGF in serum is much lower, about 150-185 pg/ml. The inventors have also found that the amount of PlGF in normotensive pregnant women falls with increasing length of gestation, such that the difference in PlGF levels between normal and pre-eclamptic pregnancies is most marked before 36 weeks' gestation. Accordingly, it is preferred to perform the method of the fifth aspect of the invention prior to 36 weeks' gestation, and preferably prior to 30 weeks' gestation. This has the added advantage of providing clinicians with advanced warning of developing hypertension, in a much more reliable manner than simply measuring blood pressure directly, as it is well known that blood pressure varies between individuals and from day to day.

As with the third and fourth aspects of the invention, the diagnostic method of the fifth aspect will conveniently comprise a specific binding assay (e.g. involving PlGF-specific immunoglobulins or effective portions thereof, and/or PlGF-specific receptor molecules, such as FLT-1 or effective portions thereof). Suitable assay techniques (e.g. ELISA, RIA, Western blotting) are known to those skilled in the art. The sample of body fluid may conveniently comprise a blood or urine sample. If desired, the method of the fifth aspect may be performed in conjunction with the method of the third and/or fourth aspects of the invention, so as to provide maximum diagnostic information to the clinician.

The diagnostic method of the third, fourth or fifth aspect of the invention may be used not

only to confirm a clinician's suspicion that a pregnant woman is suffering from a hypertensive disorder but may also, unexpectedly, be used predictively to ascertain those women who may be at increased risk of developing such a disorder before any symptoms become apparent. Clearly this is extremely advantageous as the health of such women may be monitored more closely and appropriate remedial measures taken at an early stage.

Thus, in particular, the inventors have found that the levels of PlGF are significantly lower in women who go on to develop pre-eclampsia, and that the distinction between normal women and those at risk of developing pre-eclampsia can be made at an early stage in pregnancy (e.g. by 25 weeks in nearly all cases, and as early as 16-20 weeks for some women).

In a further aspect the invention provides a kit for use in diagnosing a hypertensive disorder in a pregnant woman, the kit comprising a reagent having specific binding activity for VEGF and/or a reagent having specific binding activity for sflt, and/or a reagent having specific binding activity for PlGF and instructions for use according to the method of the third and/or fourth and/or fifth aspects of the invention. The kit will preferably comprise one or more reagents immobilised on an inert support (e.g. a microtitre plate) and conveniently may further comprise one or more control samples comprising known concentrations of VEGF and/or sflt, and/or PlGF, and appropriate packaging materials.

The most convenient form of kit will comprise a dipstick-type test which can be performed using a urine sample. Test kits of this type are familiar to those skilled in the art from pregnancy-testing kits, commercially available.

In a further aspect, the invention provides a method of making a medicament for the treatment of a hypertensive disorder in a pregnant woman, the method comprising combining a therapeutic substance, which regulates the amount and/or activity of VEGF in the woman, with a physiologically acceptable carrier, excipient or diluent. Such carriers, excipients or diluents are known to those skilled in the art and include, for example, saline solution phosphate-buffered saline and the like. The resulting medicament will typically be substantially sterile and suitable for injection or administration by other

means to the pregnant woman.

The present invention will now be further described by way of illustrative examples and with reference to the accompanying drawings, in which:

Figures 1A-1C are micrographs of sections labelled with ^{125}I-VEGF (A, B) or anti-CD34 (C), (magnification X85);

Figures 2A-2F are micrographs of sections treated with ^{125}I-VEGF (A, C-E), unlabelled VEGF (F), or anti-CD34 (B);

Figure 3 shows a number of panels (5), each with traces of representative results obtained when different samples were subjected to gel filtration chromatography on an S-200 column;

Figure 4 shows the results of polyacrylamide gel electrophoresis (PAGE) analysis of ^{125}I-VEGF cross-linked serum samples;

Figure 5 shows the results of PAGE analysis of ^{125}I-VEGF cross-linked culture supernatants;

Figure 6 shows the results of PAGE analysis of ^{125}I-VEGF cross-linked recombinant sflt;

Figures 7 and 10 are bar charts showing inhibition of ^{125}I-VEGF binding to bovine aortic endothelial (BAE) cells by various samples;

Figure 8 is a photograph of a Coomassie-stained gel;

Figure 9 shows the results of PAGE analysis of purified culture supernatants cross-linked with ^{125}-VEGF;

Figure 11 shows a graph of PlGF levels in serum (pg/ml) for pre-eclamptic (circles) and

normotensive (crosses) pregnant women, for the interval from 26 weeks' gestation to term; and

Figure 12 shows a graph of PlGF levels in serum (pg/ml) for pre-eclamptic (lozenges) and a normotensive (squares) pregnant women for the interval from 15 weeks' gestation to term.

EXAMPLE 1

Experimental Procedures

Tissue and Serum Collection

Gestational dates were calculated from the first day of the last menstrual period and were within $\pm$ 7 days of those estimated at an ultrasound scan performed in the first trimester of pregnancy. First trimester placental tissue was obtained from surgical terminations of pregnancy conducted between weeks 8 and 12 of gestation and third trimester material was obtained from normal placentae at term after Caesarean delivery. In the latter case a thin slice of tissue was taken from the maternal surface of the placenta, which contained a significant amount of extravillous trophoblast and decidua; and also a 1 cm^3 block excised from deeper in the placental cotyledon, containing only villous tissue. A sample from each area was checked by histology. Samples were either snap-frozen or fixed overnight in 10% buffered formalin and embedded in paraffin wax. Maternal serum samples were collected in non-heparinised tubes and after clotting and centrifugation, aliquotted and frozen.

Binding of ^{125}I-VEGF to Tissue Sections

Frozen tissue was sectioned and binding of ^{125}I-VEGF conducted according to the method of Shweiki *et al* (1993 J. Clin. Invest. **91**, 2235-2243). Sections were pre-incubated with 0.2% gelatin and 1 mg/ml heparin in phosphate buffered saline (PBS) for 30 min at room temperature. ^{125}I-VEGF (Amersham International plc, 1500 Ci/mMol) was then added at a concentration of 100 pM in PBS. Serial control sections contained competitor VEGF

(R&D Systems) at a concentration of 3 nM in the binding medium. After binding for 1 h at room temperature sections were washed in cold PBS, fixed in 2.5% glutaraldehyde, and washed again. Sections were air dried and autoradiographic emulsion (LM-1 emulsion; Amersham International plc) applied to the slides, which were developed after 48 hours.

Immunohistochemistry

Serial sections to those used for ^{125}I-VEGF binding were stained for CD34 (Clone QBEND/10, Serotec) to identify blood vessels. Briefly, sections were fixed for 5 minutes in 10% neutral buffered formalin and anti-CD34 was applied and detected with secondary biotin-streptavidin-horseradish peroxidase reagents from Zymed (Cambridge Biosciences). Detection was with DAB (diaminobenzidine) and no counterstaining was used.

Cloning of Soluble flt-1 (sflt-K) from the Placenta

A cDNA library prepared in λgt11 from mRNA obtained from term placenta (Clontech) was screened using a probe specific for a portion of the extracellular domain of flt-1. This probe (bases 1507 to 1779; Shibuya *et al.*, (1990 Oncogene **5**, 519-524) shows minimal homology between flt-1 and other related receptors and was obtained by PCR using the primers described by Boocock *et al.*, (1995 J. Natl. Can. Inst. **78**, 506-516). Screening was performed as described in Charnock-Jones *et al.*, (1995 Int. J. Biochem. Cell Biol. **28**, 81-89).

Numerous hybridizing plaques were identified and several of these were purified by further rounds of screening. Six of these clones, upon sequence analysis, proved to be similar to the clone described by Kendall & Thomas (EMBL accession number U01134) which encodes a soluble variant of flt-1, that we term sflt-K. The longest of these clones was fully sequenced using fluorescent dye terminators (ABI) and contained cDNA which encoded soluble flt-1. The coding portion of this cDNA from the *Eag* I site at position 189 to the linker derived *Eco* RI site at the 3' end was subcloned into the baculoviral transfer vector pVL1392 (Pharmingen) and its orientation confirmed by DNA sequencing.

DNA was prepared from this plasmid using a Qiagen miniprep and then cotransfected into Sf9 cells with baculogold DNA (Pharmingen) to allow *in vivo* site-specific recombination to occur and the subsequent generation of recombinant viruses. The recombinant viruses were plaque-purified in soft agar and the plaques were identified by visual examination under strong illumination over a dark background. High titer viral stocks were then prepared as described in the Pharmingen baculovirus manual.

Protein expression was accomplished by infecting small scale liquid cultures of approximately 100ml of Sf9 cells at density of approximately $2x10^6$ per ml with a high titer viral stock at a multiplicity of infection of between 2 and 10. Cells were grown in complete TMN-FH medium (Pharmingen) or SF 900-II serum free medium (GIBCO/BRL).

Protein Purification

Soluble receptor was purified using the method described by Kendall and Thomas (1993 Proc. Natl. Acad Sci USA **90**, 10705-10709). Conditioned medium (45ml) from the baculoviral infected Sf9 cells was clarified and then passed through a 1 ml heparin Sepharose affinity column (Hi-trap column, Pharmacia Biotech.) which had been previously equilibrated with 0.15 M NaCl in 10 mM sodium phosphate (pH 6.2). The column was washed with this buffer and also with a similar buffer containing 0.6 M NaCl. Soluble receptor was eluted from the column using 1.0 M NaCl in sodium phosphate buffer. The protein was desalted and concentrated further by centrifugal ultrafiltration (Centricon 30, Amicon Inc.).

S-200 Gel Filtration Chromatography

^{125}I labelled VEGF (Amersham International plc) was incubated at a final concentration of 15 pM with 400 µl of serum obtained from healthy male or female volunteers and pregnant women at different stages of gestation. Samples of recombinant sflt-K and villous culture supernatant were also examined. After incubation overnight at room temperature samples were analysed by gel filtration chromatography on a 12.5 ml S-200

Sephadex column (dimensions of 0.8 cm x 25 cm) as described by Hill *et al.*, (1995 J. Clin. Endocrinol. & Metab. **80**, 1822-1831). The column was equilibrated in PBS and eluted in the same buffer. Fractions (400 μl) were collected and the elution position of the ^{125}I-VEGF determined by γ counting (Packard). The column was also run using ^{125}I-VEGF in the absence of serum and also after incubation of the ^{125}I-VEGF with serum and a large excess of unlabelled VEGF (5 nM).

^{125}I-VEGF Binding in Serum

Serum samples from non-pregnant women and women in the third trimester of pregnancy were investigated (n=3 in each group). Serum (5 ml) was mixed with an equal volume of 10 mM sodium phosphate (pH 6.2) with 0.15 M NaCl and passed through a 1 ml heparin Sepharose affinity column as described above for sflt-K. The 0.6 M wash and 1.0 M eluted fractions were collected (6 ml of each), desalted and concentrated 6 fold in Centricon 50 tubes (Amicon Inc.). Crosslinking and polyacrylamide gel electrophoresis was conducted as described below.

Covalent Crosslinking

^{125}I-VEGF at a concentration of 900 pM was incubated with samples overnight at room temperature. Control experiments where undertaken with 25 ng of VEGF (Amgen) which competed with the ^{125}I-VEGF. Complexes were crosslinked with 5 mM bis(sulfosuccinimidyl)suberate (Pierce) dissolved in 5 mM Na Citrate (pH 5.0), for 5 min at room temperature. The reaction was terminated with Tris-HCl (pH 6.8) containing 10% SDS and 8% glycerol. Samples were reduced with a 2% volume of β-mercaptoethanol and subjected to electrophoresis on 4-15% gradient polyacrylamide gels (Biorad).

Binding of ^{125}I-VEGF to Bovine Aortic Endothelial (BAE) Cells

BAE cells were cultured to confluence in 24 or 48 well plates and all experiments were conducted in triplicate at 4°C. Cells were rinsed in wash buffer (PBS containing BSA 1

mg/ml and KI 10^{-7} M). ^{125}I-VEGF at a final concentration of 20 pM was added to the binding medium (DMEM/F12, HEPES 25 mM, BSA 1 mg/ml, KI 10^{7} M, heparin 10 μg/ml). To ascertain the level of non-specific binding identical incubations were performed in the presence of 2 nM unlabelled VEGF (R&D Systems). Cells were incubated for 2 h and rinsed three times with wash buffer before being solubilised in 0.5 M NaOH and ^{125}I-VEGF levels determined by γ counting (Packard).

Villous Culture and Protein Purification

First trimester villous from four patients was sorted and was washed thoroughly with PBS. Each of the villous samples was cultured in a 60mm petri dish for 24 hours, the medium was changed and the samples were cultured for a further 48 hours in 6 ml of serum-free, phenol-red free DMEM/F12. Medium alone was subjected to the same conditions as a control. Samples of the villous tissue were fixed for histological examination. Protein was purified from the supernatant using the method of Kendall & Thomas (cited previously) for sflt-K purification. Medium was pooled and loaded onto a 1 ml Hi Trap Heparin Column (Pharmacia Biotech) which was equilibrated and eluted as described above. The elutate was collected in 2 fractions of 3 ml each. Control 0.6 M wash, and 1.0 M NaCl eluted fractions were desalted in Centricon 30 tubes (Amicon Inc.) and concentrated to 500 μl. Control medium was purified by the same method. Samples were reduced with β-mercaptoethanol and subjected to electrophoresis on 4-15% gradient gels (Biorad) before being stained with Coomassie Blue.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) on Placenta

RNA was extracted according to the method of Chomczynski and Sacchi (1987 Analytical Biochem. **162**, 156-159) and cDNA primed with random hexamers (Pharmacia Biotech.) and synthesised with super reverse transcriptase (HT Biotechnology Ltd). Successful cDNA synthesis was confirmed by RT-PCR using primers for histidyl tRNA synthetase as previously described (Sharkey *et al.*, 1995 Biol. Reprod. 53, 955-962). These confirmed successful RNA extraction and cDNA synthesis. PCR primers specific for the amplification of sflt-K were 5' GCAAGGTGTGACTTTTGTTC 3' and 5'

CTTTGTGTGGTACAATC 3' (Seq. ID Nos. 1 and 2 respectively) ; the latter being after the splice site for sflt-K as described by Kendall and Thomas (cited above). This amplified a 617 bp region from position 1681 to 2297 according to the sequence in EMBL (accession number U01134). "Touch down" PCR was conducted with one cycle for 3 min at 95°C for 30 s, X°C for 30 s, 72°C for 30 s (where X = 56°C, 54°C, 52°C, 50°C and finally 48°C) for 2 cycles each. This was followed by 15 cycles where X=46°C and a final extension period of 3 min at 72°C. A negative PCR control, with no cDNA in the reaction was included.

RNase Protection Assay

The method used was essentially that of Charnock-Jones (1994 J. Reprod. & Fertil. **101**, 421-426). A flt-1-specific probe was generated which corresponded to base pairs 2014 to 2297 according to the flt-1 EMBL sequence (accession number U01134). This spans the region where sflt-K and the membrane bound form differ so that the full length probe was protected for sflt-K while a smaller 205 bp fragment of RNA was protected for full length flt-1. The antisense probe was labelled with a total of 60 μCi of $\alpha[^{32}P]$UTP (800 Ci/mmol, Amersham International plc) using the MAXIscript *in vitro* transcription kit (Ambion). The resulting probe was treated with RNase-free DNase to remove the template before being purified by preparative PAGE and eluted from the polyacrylamide. For each 30 μg sample of placental RNA, 100,000 cpm of probe was mixed and precipitated before addition of the hybridisation buffer (RPAII kit, Ambion). This mix was heat denatured and then hybridised overnight at 45°C. Excess probe was digested with RNase A and the hybridised probe precipitated with ethanol and loaded onto a 6% polyacrylamide sequencing gel.

Placental RNA samples examined were from the surface and deep regions of term placenta and from the villi of first trimester placenta collected as already described. Five samples from each group were examined. Results were expressed as a ratio of sflt-K to full length flt-1 mRNA.

Results

^{125}I-VEGF Binding Sites in the Human Placenta

Binding sites for VEGF within first trimester and term placental tissue were investigated. Frosen tissue sections from a first trimester (week 10) placenta are representative of the localisation pattern found at this stage of gestation (n=3). The results are shown in Figures 1A and 1B. Figure 1A is a dark field image with specific binding in defined regions around a villus and associated with blood vessels (v) within the villus core. Figure 1B is a dark field on bright field of the same section as in 'A'. Binding sites were not on the trophoblast layers (arrow) but just within it. Villous stroma had some low level binding visible in both 'A' and 'B'. Binding was present around the edge of the villi but not in a continuous layer.

Serial or near serial sections were immunostained with CD34 to detect blood vessels within the villous core. The results are shown in Figure 1C. The CD34 staining pattern mirrored ^{125}I-VEGF localisation and confirmed that the strong binding was to the entothelial cells of the blood vessels. In several sections light diffuse binding was detected over some areas of trophoblast and over stromal cells. First trimester decidual sections were also investigated and a diffuse binding, above the levels of non-specific controls, was observed (data not shown). This binding was specific and could be abolished by the addition of excess unlabelled VEGF.

On term placentae ^{125}I-VEGF bound within large and small villi in a pattern which was compatible with localisation to blood vessels (n=3).

The results are shown in Figures 2A-2F. Figure 2A is a dark field photograph of binding within villi in the placenta. Figure 2B is a serial section immunostained with anti-CD34, staining blood vessels within the placenta and revaling a similar localization pattern as seen in 2A. There is some light background staining around the large villus in 2B, and within the large villus the blood vessels are particularly evident as well as there being diffuse grains within the stroma.

Figure 2C is a bright field micrograph at higher magnification and Figure 2D is a

micrograph in which a dark field and a bright field image have been merged. Figure 2C reveals that ^{125}I-VEGF binding was not over the trophoblast but within the villous core (arrowed), more particularly the blood vessels (v) within the villi, and to a lesser extent the stromal core.

Figures 2E and 2F are micrographs of serial sections: 2E (dark field on bright field) shows diffuse labelling is apparent on the decidua (asterisked) and within the villi (right hand side of the micrograph). The serial section shown in Figure 2F (which had the addition of 3nM unlabelled VEGF in the binding mix) confirms that the binding seen 2E is specific. The background level of ^{125}I-VEGF binding seen in 2F is representative of background levels found in control sections for Figures 1 and 2.

(Magnification: 2A, 2B, 2E, 2F = X80; 2C, 2D = X330).

sflt-1 Cloned from the placenta

Six out of the 15 clones obtained from a placental cDNA library were sflt-K as opposed to full length flt-1. For sflt-K additional 5' sequence to that published by Kendall and Thomas (EMBL accession number U01134) was obtained and was 5' CGGAGCGCGCCAG 3' (Seq. ID No. 3). At the 3' end a new poly A termination site was found. The translation termination site for sflt-1 ends at bp 2313 in the Kendall and Thomas sequence and the new poly A tail was found from bp 2331 onwards.

A Factor in Pregnancy Serum Binds VEGF

An S-200 Sephadex column was used to separate ^{125}I-VEGF linked to serum samples (n=3 from each group), cultured villous supernatant (n=3) and recombinant sflt-K (n=2). ^{125}I-VEGF in PBS was placed on the column to provide a baseline and the results (shown in Figure 3) indicated that free VEGF was detected around fraction 30 and another small peak occurred around fraction 15. Binding at faction 15 was found to occur consistently but was of a low affinity as it was competed for by other binding proteins (Fig. 3). Ligand blotting studies revealed that ^{125}I-VEGF binds to purified BSA (data not shown).

The ^{125}I-VEGF is supplied in BSA and therefore it is highly probable that the binding seen at faction 15 is to BSA. The three samples of male serum and non-pregnant female serum also gave a peak around fraction 15 as indicated by the arrow labelled BSA/VEGF (Fig. 3, Male). In serum samples from early pregnancy (week 12) and at term, ^{125}I-VEGF binds to a larger serum protein and this comes off the column around fractions 10-11 (Fig. 3, large arrow). This binding competes with the BSA/VEGF peak. At term, one out of the three samples had very high levels of binding in this fraction. Samples from cultured first trimester villous supernatant (V.S.) gave a peak at fraction 10-11 and recombinant sflt-K at fraction 11 (bottom panel). Control medium (DMEM/F12) for the villous culture experiments gave only a small peak at fraction 15.

^{125}I-VEGF Binding in Serum

The method used by Kendall and Thomas (cited above) for the purification of recombinant sflt-K was applied to serum samples. The results are shown in Figure 4.

The method of crosslinking of human serum with ^{125}I-VEGF and gel electrophoresis was as follows: non-pregnant and a term pregnant serum were placed on a heparin column and the 0.6 M control and 1.0 M NaCl fractions collected. Samples were incubated with ^{125}I-VEGF and crosslinked before PAGE. In Figure 4, the gel was loaded as follows: Lane 1, 1.0 M NaCl fraction of term pregnant serum (with bound ^{125}I-VEGF); Lane 2, 1.0 M NaCl fraction of non-pregnant serum (with only unbound VEGF); Lane 3, unlabelled VEGF; Lane 4, 0.6 M NaCl fraction from term pregnant serum; Lane 5, 0.6 M NaCl fraction from non-pregnant serum.

In all three serum samples from pregnant women the 1.0 M NaCl fraction contained a VEGF binding protein which when complexed with ^{125}I-VEGF gave a molecular weight just over 250 kDa (lane 1). This protein was not present in the non-pregnant samples (lane 2) and could be competed for with unlabelled VEGF (Lane 3). The control 0.6 M fraction from pregnant serum (Lane 4) had a small amount of bound ^{125}I-VEGF at the same molecular weight as the 1.0 M fraction. It is likely that this is the same binding protein and that a small amount was eluted from the column in the washes. No binding

was evident with the 0.6 M non-pregnant samples (Lane 5). Unbound VEGF was detectable as both a monomer and as a dimeric (at 23 and 46 KD respectively).

Placental Villi Produce a Soluble Factor which Binds VEGF in a Similar Way to Recombinant sflt-K

First trimester villus tissue was cultured in serum free medium as described and the resulting medium (villus supernatant, "V.S.") was incubated with ^{125}I-VEGF overnight and crosslinked. Samples were reduced and examined after gel electrophoresis (Fig. 5). VEGF/V.S. bands were found at approximately 250 and 160 kDa, which could be displaced by the addition of unlabelled ("cold") VEGF. Villous cultures were thus found to produce a soluble factor(s) capable of binding VEGF. Recombinant sflt-K was also crosslinked with ^{125}I-VEGF and this resulted in bands, of similar molecular weight to those of the villus cultures (Fig. 6). Again, unbound monomeric or dimeric VEGF is apparent at about 23 and 46 KD respectively.

Soluble Villous Factor and Recombinant sflt-K Both Inhibit Binding of ^{125}I-VEGF to Bovine Aortic Endothelial (BAE) Cells

The binding of ^{125}I-VEGF to BAE cells was used to assay for the inhibitory activity of factors binding VEGF as follows: first trimester villous from three individuals (1.V.S. to 3.V.S.) was cultured for 48 h in 6 ml of media and 5 μl or 25 μl of this supernatant used in a 500 μl ^{125}I-VEGF binding assay conducted in the presence of heparin. Results are presented (in Figure 7) as the percentage of control.

Addition of 2 nM unlabelled VEGF reduced binding to below 5% of the control. The heparin purified recombinant sflt-K inhibited binding of ^{125}I-VEGF to the BAE cells. In all cases the three villous supernatants showed dose-dependent inhibition of binding, 5μl/well having less effect than 25 μl/well. There was some variation in the activity of the three culture supernatants. Since the concentration of recombinant sflt-K added to the assay was not known, no quantitative comparison could be made with the VEGF inhibition by the villous supernatant.

Soluble Protein Purified from Villous Culture Supernatant Under Conditions Used to Purify sflt-K

The method of purifying recombinant sflt-K used by Kendall and Thomas was applied to the villous culture supernatant. Villous supernatant was loaded onto a heparin column, washed with 0.6 M NaCl and eluted with 1.0 M NaCl. Samples were desalted and concentrated before being separated by polyacryamide gel electrophoresis and stained with Coomassie Blue. A photograph of the gel is shown in Figure 8. In Figure 8, lanes 1 and 2 (numbering from the left) are MW standard markers, lanes 3 ("1.0(1)") and 4 ("1.0(2)") are the first and second fractions (respectively) collected when the heparin column was eluted with 1.0M NaCl. Lane 5 ("0.6") contains the fraction collected in the 0.6M NaCl wash prior to elution.

The first fraction eluted at 1.0 M NaCl gave a strong band at around 90 kDa (Fig. 8); this agreed with the result found by Kendall and Thomas. No bands were evident in the second fraction or in the 0.6 M wash. Control medium eluted with 1.0 M NaCl gave no bands using Coomassie Blue (data not shown).

Heparin Purified Villous Supernatant Retains ^{125}I-VEGF Binding Activity

When crosslinked with ^{125}I-VEGF both fractions from the 1.0 M NaCl elution of villous supernatant were found to have binding potential.

The results are shown in Figure 9.

In Figure 9, lanes 1 and 2 of the gel were loaded with the first and second fractions of the 1.0M NaCl eluate, pre-incubated with ^{125}I-VEGF. Lane 3 was loaded with the 0.6M NaCl washing, similarly treated, and Lane 4 is the control containing 1.0M NaCl-eluted medium rather than culture supernatant.

The first fraction gave a band predominantly in the 250 kDa range, which is consistent with high concentrations of sflt-K forming predominantly dimers with VEGF (Fig. 9, lane

1). Fraction 2 of the 1.0 M elution gave 250 kDa and 160 kDa bands consistent with lower concentrations of sflt-K binding as both a dimer and a monomer to the VEGF. Control fractions had very little binding activity (Fig. 9, lanes 3 and 4).

This purified material was very effective at inhibiting the binding of ^{125}I-VEGF to BAE cells. In assays performed as described previously, addition of a small amount (0.5 μl) was sufficient to have an effect and 2μl reduced binding to control levels. The results are shown as a bar chart in Figure 10. HPVS refers to heparin-purified villous culture supernatants (0.5, 2.0 and 8.0μl). The assay volume was 200μl.

Placenta Produces a Significant Amount of sflt-K Throughout Pregnancy

RT-PCR revealed that the mRNA for sflt-K was present in the placenta throughout pregnancy. An RNase protection assay was designed to determine levels of both full length flt-1 and sflt-K in the placenta. The RNase protection assay detected bands for both full length flt-1 and sflt-K after only one day's autoradiography. This suggested that there was a significant amount of mRNA present for both these proteins. In all samples the full length flt-1 band was stronger than that for sflt-K. The ratio of sflt-K to full length was approximately 0.45 in all three sampling areas.

Discussion

Previous research has investigated the production and localisation of VEGF and its receptors in the human placenta (Cooper *et al.*, 1995 J. Reprod. & Fertil. **106**, 205-213; Ahmed *et al.*, 1995 Growth Factors **12**, 235-243; Clark *et al*, 1996 Hum. Reprod. **11**, 1090-1098; Vuckovic *et al.*, 1996 J. Anat. **188**, 361-366). The present inventors have used ligand binding to clarify where the VEGF binding sites are in the human placenta, and have characterised a soluble VEGF binding protein which is produced by the placental villi, can be identified in maternal circulation and is indistinguishable from sflt-K (Kendall & Thomas 1993 Proc. Natl. Acad Sci USA **90** 10705-10709) by a number of criteria.

Ligand binding was performed on first and third trimester human placentae. ^{125}I-VEGF

was found to bind principally to the fetal blood vessels within the villi with only isolated areas of trophoblast showing low level binding (Fig. 1 and 2). Under the conditions used it is likely that the ^{125}I-VEGF would have bound to both flt-1 and KDR although estimates of the K_D values for these receptors of VEGF vary dramatically (Olander *et al.*, 1991 Biochem. Biophys. Res. Comm. **176**, 68-76; Waltenberger *et al.*, 1994 J. Biol. Chem. **269**, 26988-26995; Seetharam *et al.*, 1995 Oncogene **10**, 135-147; Vaisman *et al.*, 1990 J. Biol. Chem. **265**, 19461-19466). There does however seem to be a consensus that the flt-1 high affinity sites have a dissociation constant of between 1 and 16 pM and the dissociation constants of the lower affinity KDR sites are between 10-100x that of flt-1.

Both flt-1 and KDR-like immunoreactivity has been localised to the fetal endothelial cells of the placental villi throughout pregnancy (Clark *et al.*, 1996 Hum. Reprod. **11**, 1090-1098; Vuckovic *et al.*,1996 J. Anat. **188**, 361-366) and the binding sites reported here confirm these results and show that fetal endothelial cells have specific high affinity receptors for VEGF. This is in good agreement with what is widely found in other tissues (Jakeman *et al.*, 1993 Endocrinology **133**, 848-859). In contrast to the well-defined ^{125}I-VEGF binding to the endothelial cells, some diffuse, but specific binding was detected over the decidua, villous stroma and in some regions over villous trophoblast. It appears that some ^{125}I-VEGF is retained in these areas and this can be displaced by excess unlabelled VEGF. It is possible that this binding is due to the association of a secreted soluble VEGF receptor which itself interacts with extracellular matrix.

A striking feature of the present study was the lack of binding in association with the extravillous trophoblast. Clark *et al.*, (1996 Hum. Reprod. **11**, 1090-1098) used in *in situ* hybridisation probe specific for the extracellular domain of flt-1 and detected very strong signal over the extravillous trophoblast cells throughout pregnancy. Immunohistochemical localisation with antibodies directed against the intracellular carboxyl terminus of flt-1 indicated that at least some of the flt-1 produced by these cells is membrane bound (Clark *et al.*, cited above; Ahmed *et al.*, 1995 Growth Factors **12**, 235-243; Cooper *et al.*, 1995 J. Reprod. & Fertil. **105**, 205-213). However, the lack of VEGF binding would suggest that either the mRNA detected by *in situ* hybridisation is not translated or that the majority of the flt-1 produced by these cells is a secreted form which is not retained on the surface

of the extravillous trophoblast cells. The existence of such variants of flt-1 have been reported (Kendall & Thomas 1993 Proc. Natl. Acad Sci USA **90** 10705-10709; Boocock *et al.*, 1995 J. Natl. Can. Inst. **78**, 506-516), but there has been little or no evidence hitherto that such molecules are produced in substantial quantities *in vivo*.

Reverse transcriptase-PCR using primers which specifically amplify sflt-K confirmed that there was mRNA encoding sflt-K in the placenta throughout pregnancy. Furthermore RNase protection assays revealed that there was significant amounts of both full length flt-1 and sflt-K mRNA present in the placenta. Although there was more full length flt-1 mRNA detected, the mRNA encoding sflt-K was also readily detectable. As yet it is not known whether there are differences in the half lives of these two species or whether the ratio observed for the mRNA is reflected in the protein levels. Analysis of superficial and deep samples of placenta (i.e. tissue samples with and without substantial quantities of extravillous trophoblast cells present) showed that the ratio of flt:sflt-K mRNA was the same. This suggests that in the first trimester as well as at term the villous trophoblast is an important source of sflt-K mRNA.

To determine whether the placenta is capable of translating this mRNA and secreting a protein with the characteristics of a soluble VEGF receptor, first trimester villi were cultured in serum free media and the supernatant analysed. Incubation of the villous supernatant with ^{125}I-VEGF and subsequent fractionation by gel filtration chromatography showed a binding peak that was similar to that observed when recombinant sflt-K was used (Fig. 3). Crosslinking to ^{125}I-VEGF and gel electrophoresis revealed the complexes of approximately 250 and 160 kDa. Crosslinking studies using recombinant sflt-K produced complexes of a similar molecular weight to those observed using secreted villous protein (Fig. 5 and 6). The sizes of these complexes are similar to those previously reported by Kendall & Thomas and not inconsistent with the presence of receptor dimers. To further characterise the soluble villous protein an attempt was made to purify the activity using the method developed by Kendall & Thomas (1993 Proc. Natl. Acad Sci USA **90**, 10705-10709) for the purification of the recombinant protein. This relies on heparin affinity chromatography and after washing with 0.6 M NaCl the sflt-K is eluted at 1.0 M NaCl. When this procedure was applied to villous culture supernatant the binding activity was

recovered in the 1.0 M NaCl fraction (Fig. 5). The predominant band (as detected by coomassie staining following PAGE) was at 90 kDa. This is very similar to that reported by Kendall and Thomas for recombinant sflt-K (85-90 kDa). This is however the first report of the natural occurrence of such a protein.

The data suggested that the placental villous was producing a secreted form of flt-1. To investigate whether this soluble villous protein was biologically active and could interfere with the binding of VEGF to endothelial cells it was tested in a binding assay. The results clearly demonstrated that relatively small amounts could interfere with VEGF binding to endothelial cells (Fig. 7). Furthermore, the secreted villous protein was indistinguishable from recombinant sflt-K using heparin affinity, gel filtration chromatography, and cross-linking, as well as both being potent inhibitors of ^{125}I-VEGF binding to endothelial cells.

The sflt mRNAs identified to date arise by alternative splicing (Kendall & Thomas 1993 Proc. Natl. Acad Sci USA **90**, 10705-10709; Boocock *et al.*, 1995 J. Natl. Can. Inst. **78**, 506-516): understanding the mechanisms by which this splicing is controlled will have important consequences for the biological actions of VEGF and hence angiogenesis.

In the placenta the villi are in direct contact with maternal blood, so proteins secreted by villous trophoblast may be detectable or active in maternal circulation. The inventors investigated whether VEGF binding activity could be identified in the serum of pregnant women. Using the binding and gel filtration assay described above serum from women in the first trimester of pregnancy and at term had binding activity which was not detectable in the serum from non-pregnant women or males (Fig. 3). A VEGF binding protein was also purified from term pregnant serum by the method used by Kendall and Thomas for sflt-K but was not detected in non-pregnant serum (Fig. 4).

It has been suggested that α 2 macroglobulin, present in high levels in human serum, binds and inactivates VEGF (Soker *et al.*, 1993 J. Biol. Chem. **268**, 7685-7691), however, the column fractionation and crosslinking studies conducted here did not detect this binding in any of the serum samples tested (male, non-pregnant and pregnant female). Neither could it be shown that VEGF was inactivated by α 2 macroglobulin.

The inventors conclude that the placenta produces a VEGF-binding protein that is indistinguishable from sflt-K. Furthermore, large amounts of this protein are released into the maternal circulation. *In vitro* recombinant forms of this protein have been shown to inhibit the action of VEGF (Kendall & Thomas, 1993 Proc. Natl. Acad Sci USA **90**, 10705-10709). Of greater significance is the observation that artificially engineered VEGF-receptor chimeric proteins can block VEGF induced retinal neovascularisation (Aiello *et al.*, 1995 Proc. Natl. Acad. Sci. USA **92**, 10457-10461). This indicates that such proteins could play a role in the regulation of VEGF action *in vivo*. Thus it is possible that the sflt-K produced by the trophoblast provides a mechanism for regulating the action of VEGF in the decidua. This is a site where large amounts of VEGF are produced by the macrophages in Nitabuch's stria during the first trimester of pregnancy; but where the process of vascular transformation is required rather than angiogenesis.

EXAMPLE 2

The present inventors developed the hypothesis that, in pregnancy, the level of bioactive VEGF is of great importance and is tightly regulated by the placental production of a soluble receptor for VEGF. Therefore, perturbation of this system, either by production of excess VEGF or insufficient VEGF antagonist (soluble receptor) could lead to homeostasis problems which may cause some of the placental pathology observed in pre-eclampsia. To investigate this possibility *in vivo* experiments were performed.

Pregnant mice were injected with 1.5μg of VEGF165. VEGF165 is so-named because each polypeptide chain present in the dimeric molecule comprises 165 amino acids. (The other most common form of VEGF is VEGF121). VEGF165 was provided by colleagues of the inventors at Amgen and was prepared by expression of the relevant nucleotide sequence (reviewed by Ferrara *et al.*, 1992 Endocrine Reviews **13**, 1) in *E. coli*. However, equivalent VEGF165 is commercially available from a number of sources, including R & D Laboratories (Abingdon, Oxford, UK, catalogue number 293/VE).

The mice were injected daily, by the intraperitoneal route, between days 8 and 17 of gestation (day 1 = day of vaginal plug formation). Control mice were injected with

vehicle (PBS) alone on the same days. Histological examination of the placentae from the treated animals revealed several important features:

1) Marked increase in fibrin deposition;
2) Increase in endothelial cell dirsuption;
3) General disruption of the labyrinthine layer of the placenta which, in many cases, would have lead to placental failure;
4) Significant increase in the number of resorption sites in the treated animals compared to the controls ($p < 0.05$);
5) Highly significant reduction in the embryonic weights of the mice in the treated group ($p < 0.001$), whilst there was no difference in placental weights.

The results are shown in more detail in Tables 1 and 2 below.

Table 1: Number of Resorption Sites

Number of Resorption Sites	0	1	2	3	4	5
Control group	3	1	0	0	0	0
VEGF-treated group	0	0	1	1	2	1

The table shows the number of resorption sites in the uterus (where the embryo has died and the embryo and placenta are being broken down and resorbed), in pregnant mice treated either with vehicle (PBS, control group) or with VEGF165 in PBS. There is a statistically significant difference between the results for the two groups.

Table 2 below shows the weights of embryos in pregnant mice treated either with vehicle (PBS, control group) or with VEGF165 in PBS. There is a statistically significant difference between the results for the two groups.

The inventors conclude from these data that shifting the relative level of bioactive VEGF by administration of recombinant VEGF resulted in the appearance of many of the features of pre-eclampsia in the treated mice.

Table 2 : Embryo weights

Variable	Control group	VEGF165 group
Mean weight (g)	0.53	0.46
Std. deviation	0.05	0.09
No. of observations	29	39
t Statistic	4.04	
Deg. freedom	66	
Significance	$p<0.001$	

These data strongly support the suggestion that perturbations in the VEGF/soluble receptor system cause some of the features of pre-eclampsia in humans. The findings therefore provide a way of diagnosing the condition prior to the onset of clinical symptoms, and treatment to restore the balance of VEGF/soluble receptor by reducing the amount of bioactive VEGF (or possibly, in certain cases, increasing the level of bioactive VEGF). It also provides for the possibility of screening for susceptibility for developing pre-eclampsia. It will be appreciated that the inventors have also developed an animal model for pre-eclampsia, the animal having abnormal levels (typically abnormally high levels) of bioactive VEGF, which could be as a result of genetic engineering (e.g. using transgenic animals with the VEGF gene overexpressed) or as a result of administering substances which affect the level of bioactive VEGF. Such animals (preferably laboratory mammals, conveniently mice or rats) could then be used in trials to discover new substances with efficacy in the treatment of pre-eclampsia. This represents a significant development, as hitherto no suitable animal model has been found.

EXAMPLE 3

Placental Growth Factor (PlGF) during Pregnancy

PlGF is a dimeric, secreted factor capable of promoting endothelial cell proliferation *in vitro* which is structurally related to VEGF (Maglione *et al.*, 1991 Proc. Natl. Acad. Sci. **88**, 9267-9271). Three alternatively spliced variants have been identified with PlGF-2 having affinity for heparin (Cao *et al.*, 1997 J. Biol. Chem. **271**, 3154-3162). Recombinant

PlGF-1 produced by overexpressing eukaryotic cells is highly active on endothelial cells in chemotactic, mitogenic and angiogenic assays (Ziche *et al.*, 1997 Lab. Invest. **76**, 517-531). Human umbilical vein endothelial cells display two classes of binding sites for PlGF homodimers. The high affinity binding is to FLT-1 while the lower affinity receptor is yet to be identified (Park *et al.*, 1994 J. Biol. Chem. **269**, 25,646-25,654; Clauss *et al.*, 1996 J. Biol. Chem. **271**, 17629-17634). PlGF/VEGF heterodimers have been found which bind to the KDR receptor (Cao *et al.*, 1996). PlGF and VEGF can thus act in unison on both monocyte and endothelial cells (Clauss *et al.*, 1996). The ability of PlGF to bind the VEGF receptors indicates that it may be important in regulating the VEGF system as well as inducing angiogenesis itself. The inventors have investigated a): the localisation of PlGF and VEGF in the placenta to determine whether there is any co-localisation and thus whether heterodimer formation is likely to occur, and b): the levels of PlGF in the plasma of pregnant women before the onset of pre-eclampsia (levels were also determined in gestationally age-matched control women who did not subsequently develop pre-eclampsia).

In situ hybridisation was performed as previously described (Clark *et al.*, 1996 cited above) with probes specific for VEGF-A and PlGF. Plasma samples were collected in EDTA tubes from normotensive and pre-eclamptic women and were matched for gestational age (within 7 days). Plasma samples were also collected from five non-pregnant women. Diagnosis of pre-eclampsia required a blood pressure of at least 140/90 mmHg, or an increase in diastolic blood pressure of greater than 25 mmHg. These patients also developed proteinurea which was at least 2+ of protein of a catheterized specimen or at least 300 mg in a 24 hr collection as previously detailed by Sharkey *et al.* (1996 Eur. J. Clin. Invest., **26**, 1182-1185). The samples were analysed in duplicate on a R & D ELISA plate for PlGF. Recovery of recombinant PlGF added to the normotensive plasma was 94%. The assay measured down to 7 pg/ml of PlGF.

A summary of the *in situ* hybridisation findings is given in Table 3. No co-localisation of PlGF or VEGF was found in the placenta indicating that heterodimers are unlikely to be formed. Thus the PlGF from the placenta will only bind the FLT-1 receptor.

Table 3. Summary of PlGF and VEGF *in situ* hybridisation results.

	PlGF Wks 6-12	PlGF Wks 14-22	PlGF Term	VEGF Wk 6-Term
Villous Troph.	++	-	+++	-
Villous Stroma	-	-	-	+
EVT Cells	+++	+++	+++	-
Maternal Glands	-	-	-	+++
Non-EVT Maternal Cell	-	-	-	+++

ELISA analysis of plasma samples are given in Fig. 11. In two of the non-pregnant women the levels of PlGF were below the limit of detection of 7 pg/ml. In the other three non-pregnant women ("Non-P" in Fig. 11) levels were from 7-8 pg/ml. The levels of PlGF in the plasma of pre-eclamptic women (circle symbols in Fig. 11) ranged from 21-1093 pg/ml but with only one sample over 257 pg/ml. The average for the pre-eclamptic plasma was 185 pg/ml. Interestingly the one pre-eclamptic woman with the very high level of PlGF (1093 pg/ml) had the lowest VEGF levels as measured by the method of Sharkey *et al.* (1996) and was thus more like the normotensive group. Control pregnant levels (crosses in Fig. 11) ranged from 182-1007 pg/ml with an average of 504 pg/ml. The difference in the serum levels of PlGF in the normotensive and pre-eclamptic women is statistically extremely significant ($p<0.0003$). In the control group there is a significant decline in the circulating PlGF levels as gestation progresses. This change is not apparent in the pre-eclamptic group. Thus an early test may be more powerful than one later in gestation.

Blood was collected from pregnant women at different stages of gestation (from approximately 15 weeks until term) and the plasma separated and stored until subsequent analysis. Levels of PlGF were determined using an ELISA. These data show that there was a marked rise in PlGF levels in the serum of blood in women who did not develop pre-eclampsia. This rise began early in the second trimester of gestation. In patients who

subsequently went on to develop pre-eclampsia there was no such rise in the blood levels of PlGF. This marked difference is apparent before the onset of pre-eclampsia and therefore determination of the circulating levels of PlGF is predictive for the women who subsequently develop pre-eclampsia. The data shown in Figure 12 indicate that these levels become predictive from about the 20th week of gestation.

It is thus evident that circulating PlGF will be able to bind both soluble and membrane bound FLT-1 receptor. This in turn will influence the dynamics of VEGF binding to both FLT-1 and KDR. The difference between PlGF levels between normotensive and pre-eclamptic women is very marked and is evident before the onset of the disease so tests at 20-25 weeks' gestation, or possibly even earlier (e.g. 16-19 weeks), could be predictive and allow identification of women at increased risk of developing pre-eclampsia.

Given that PlGF and VEGF both interact with FLT and soluble FLT, a diagnostic and/or predictive test could be based on the determination of the amount of any one of PlGF, VEGF or sFLT in the women, preferably a measurement of any two thereof, and most preferably a measurement of all three substances. It is possible that the ratio of any two or more of the substances may be more informative or predictive than the absolute levels of the substances.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Cambridge University Technical Services Limited
(B) STREET: The Old Schools. Trinity lane
(C) CITY: Cambridge
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): CB2 1TS

(ii) TITLE OF INVENTION: Diagnosis and Treatment of Pathological Pregnancies

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTTTGTGTGG TACAATC 17

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGGAGCGCGC CAG 13

Claims

1. A method for treating a hypertensive disorder in a pregnant woman, the method comprising administering to the pregnant woman an amount of a therapeutic substance which regulates the amount, and/or activity, of VEGF in the woman effective to ameliorate the hypertensive disorder.

2. A method according to claim 1, wherein the method reduces the amount, and/or activity, of VEGF in the woman.

3. A method according to claim 1 or 2, wherein the therapeutic substance inhibits binding of VEGF to cell-membrane VEGF receptors on target cells.

4. A method according to any one of the preceding claims, wherein the therapeutic substance inhibits one or more steps following binding of VEGF to its cell-membrane receptor.

5. A method according to any one of the preceding claims, wherein the therapeutic substance has specific binding activity for VEGF or for a VEGF receptor.

6. A method according to any one of the preceding claims wherein the therapeutic substance comprises a polypeptide.

7. A method according to any one of the preceding claims, wherein the therapeutic substance comprises a fusion protein or chimeric polypeptide.

8. A method according to any one of the preceding claims, wherein the therapeutic substance comprises an immunoglobulin molecule or an effective portion thereof.

9. A method according to any one of the preceding claims, wherein the therapeutic substance comprises a soluble form of FLT-1.

10. A method according to any one of the preceding claims, wherein the therapeutic substance comprises a VEGF-specific immunoglobulin molecule or an effective portion thereof.

11. A method according to any one of the preceding claims, wherein the therapeutic substance comprises Placental Growth Factor (PlGF) or a portion thereof which retains specific binding activity for FLT-1.

12. Use of a therapeutic substance which regulates the amount and/or activity of VEGF in a human subject, in the manufacture of a medicament to treat a hypertensive disorder in a pregnant woman.

13. Use according to claim 12, in the manufacture of a medicament for use in the method of any one of claims 1-11.

14. A method of diagnosing a hypertensive disorder in a pregnant woman, the method comprising: obtaining a sample of body fluid from the woman; and determining, qualitatively or quantitatively, the amount of VEGF and/or VEGF antagonist in the sample.

15. A method according to claim 14, the method comprising: obtaining a sample of body fluid from the woman; and determining, qualitatively or quantitatively, the amount of soluble VEGF receptor in the sample.

16. A method according to claim 15, comprising determining the amount of sFLT in the sample.

17. A method of diagnosing, or predicting develpoment of, a hypertensive disorder in a pregnant woman, the method comprising: obtaining a sample of body fluid from the woman; and determining, qualitatively or quantitatively, the amount of Placental Growth Factor in the sample.

18. A method according to claim 17, further comprising determining qualitatively or

quantitatuvely, the amount of VEGF and/or the amount of soluble VEGF receptor in a sample of body fluid from the woman.

19. A method according to any one of claims 14-18, wherein the sample comprises blood or urine.

20. A method according to any one of claims 14-19, comprising the use of a specific binding partner having specific binding activity for at least one of VEGF, soluble VEGF receptor, or PlGF.

21. A method according to any one of claims 14-20, comprising an ELISA, RIA or Western blot assay.

22. A kit for diagnosing a hypertensive disorder in a pregnant woman, the kit comprising a reagent having specific binding activity for at least one of the following: VEGF, soluble VEGF receptor and PlGF; and instructions for use in the method of any one of claims 14-21.

23. A kit according to claim 22, comprising one or more reagents immobilised upon an inert solid support, or one or more control samples comprising a known concentration of VEGF, or soluble VEGF receptor, or PlGF.

24. A method of making a medicament for use in the treatment of a hypertensive disorder in a pregnant woman, the method comprising combining a therapeutic substance, which regulates the amount and/or activity of VEGF in the woman, with a physiologically acceptable carrier, excipient or diluent.

25. A method according to claim 24, further comprising the step of dividing the medicament into unitary doses.

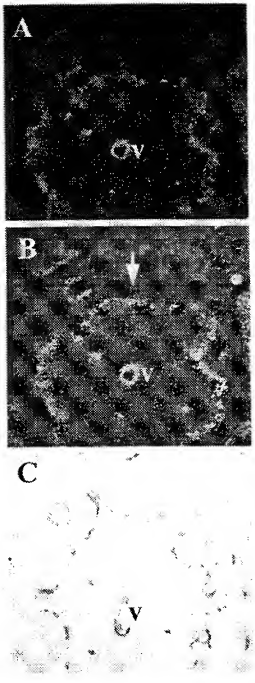


Fig. 1

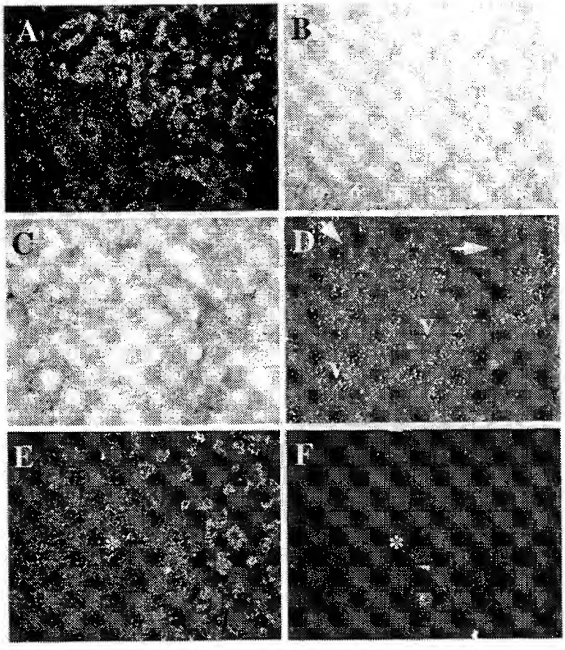


Fig. 2

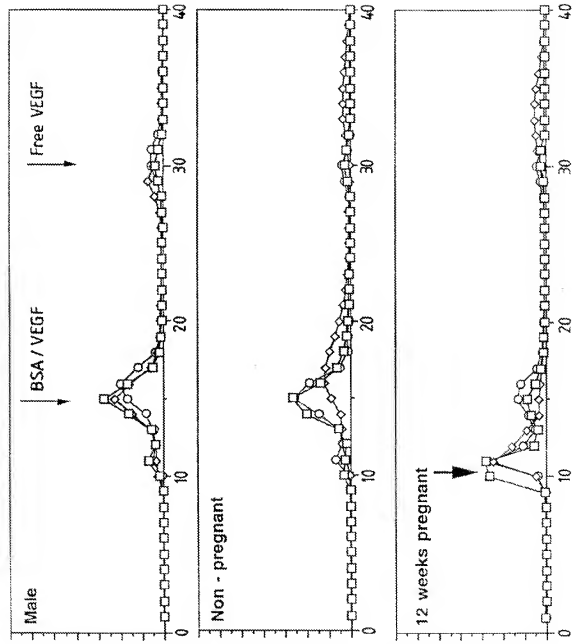

Male
BSA / VEGF
Free VEGF
Non - pregnant
12 weeks pregnant
% 125I-VEGF recovered
0
10
20
30
40
50
60
70

Fig. 3
Sheet 1

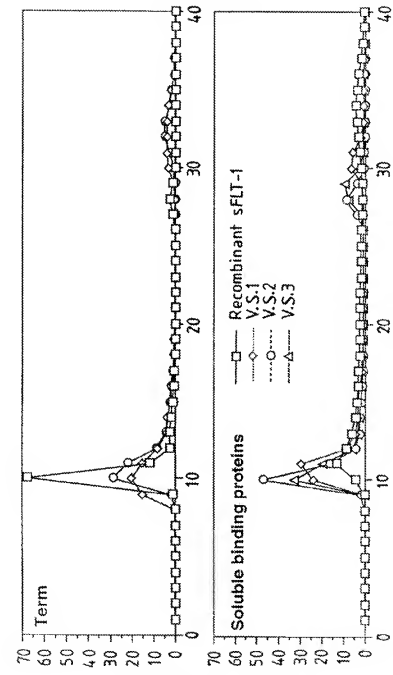

Term
Soluble binding proteins
Recombinant sFLT-1
V.S.1
V.S.2
V.S.3
% 125I-VEGF recovered
Fraction number
0
10
20
30
40
70
60
50
40
30
20
10
0

Fig. 3
Sheet 2

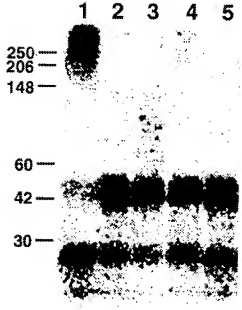


Fig. 4

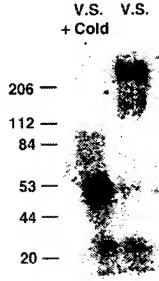
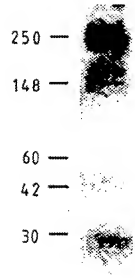


Fig. 5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148
60
42
30

Fig. 6

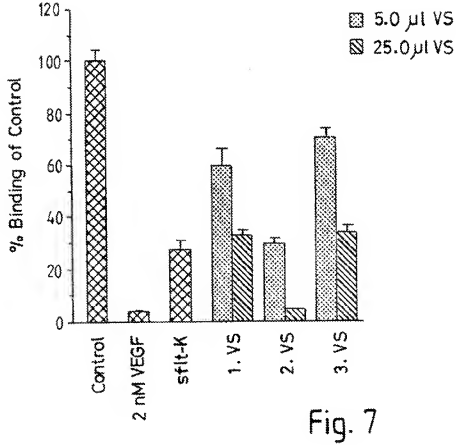
5.0 µl VS
25.0 µl VS
% Binding of Control
120
100
80
60
40
20
0
Control
2 nM VEGF
sflt-K
1. VS
2. VS
3. VS

Fig. 7

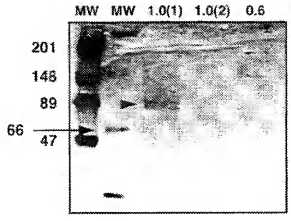

Fig. 8

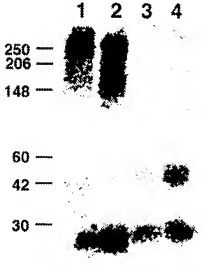

Fig. 9

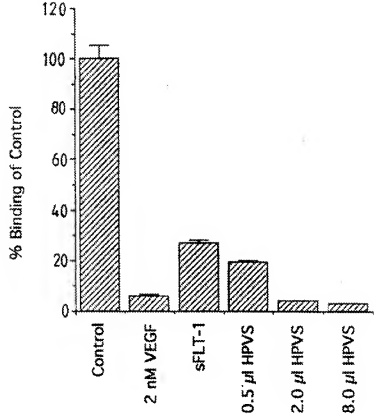
% Binding of Control
120
100
80
60
40
20
0
Control
2 nM VEGF
sFLT-1
0.5 µl HPVS
2.0 µl HPVS
8.0 µl HPVS

Fig. 10

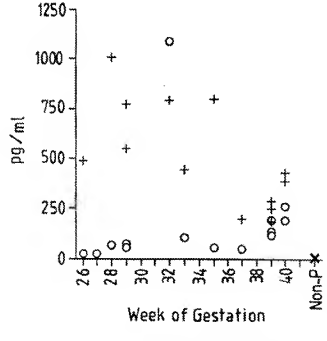
pg/ml
1250
1000
750
500
250
0
26
28
30
32
34
36
38
40
Non-P
Week of Gestation

Fig. 11

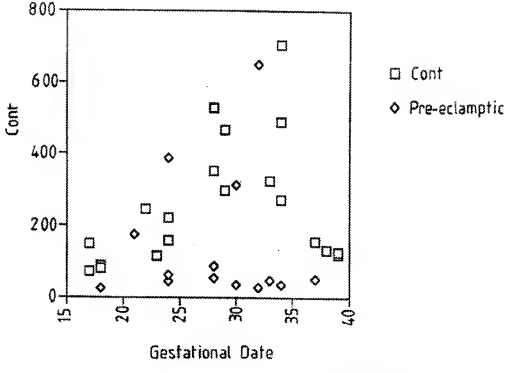
800
600
400
200
0
Cont
15
20
25
30
35
40
Gestational Date
Cont
Pre-eclamptic

Fig. 12

INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/GB 97/03519

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 A61K38/17 A61K38/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 10202 A (GENENTECH INC.) 11 May 1994 cited in the application see abstract see page 17, line 4 see page 2, line 29 - page 3, line 23 see page 8, line 28 - line 31 see page 10, line 22 - page 13, line 3 see page 15, line 1 - page 17, line 5	1-10,12, 13,24,25
Y	--- -/--	16,20-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
7 April 1998	21.04.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Alvarez Alvarez, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ational Application No
PCT/GB 97/03519

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	P. N. BAKER ET AL.: "Elevated serum levels of vascular endothelial growth factor in patients with preeclampsia" OBSTETRICS & GYNECOLOGY, vol. 86, no. 5, November 1995, NY, USA, pages 815-821, XP002061604 see the whole document	14,15,19
Y		1-3,5, 12,13, 16,20-25
Y	J.V.S. MAECK ET AL.: "Heparin in the treatment of toxemia of pregnancy" AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY, vol. 55, no. 1, January 1948, pages 326-331, XP002061605 see figures 1,2 see "Discussion"	1-3,5, 12,13
A	WO 95 33050 A (LYNXVALE LIMITED ET AL.) 7 December 1995 cited in the application	
A	V. J. RAPPAPORT ET AL.: "Anti-vascular endothelial cell antibodies in severe preeclampsia" AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY, vol. 162, no. 1, January 1990, pages 138-146, XP002061606 see "Comment" pages 143-145	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna ial Application No
PCT/GB 97/03519

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9410202 A	11-05-94	AU 2928992 A	24-05-94
		BG 99605 A	29-02-96
		BR 9207175 A	12-12-95
		EP 0666868 A	16-08-95
		FI 951987 A	26-04-95
		JP 8502514 T	19-03-96
		NO 951609 A	27-04-95
		SK 55195 A	09-08-95
WO 9533050 A	07-12-95	AU 2535495 A	21-12-95
		CA 2191071 A	07-12-95
		EP 0783571 A	16-07-97